IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Fung et al.	Confirmation No.: 4825
Serial No.: 10/583,927 (National Stage of Int'l Application No. PCT/US04/43501)	Art Unit: To be assigned
Int'l Filing Date: December 23, 2004	Examiner: To be assigned
For: NOVEL ANTI-IL-13 ANTIBODIES AND USES THEREOF	Attorney Docket No.: 12279-187-999

PETITION UNDER 37 C.F.R. § 1.47(a)

Mail Stop PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Pursuant to 37 C.F.R. § 1.47(a), Sek Chung Fung, Matthew Moyle, Mason Lu, Sanjaya Singh, and Dan Huang, inventors of the above-identified application, hereby petition for entry of the Declaration that they have executed on behalf of themselves and on behalf of Changning Yan, a joint inventor who cannot be reached. In support of this petition and pursuant to 37 C.F.R. § 1.47(a), Applicants submit herewith a Declaration of Hong-Van M. Le in Support of Petition Submitted Under 37 CFR § 1.47(a), including proof of the pertinent facts regarding the inability to reach Changning Yan to have her sign a Declaration in connection with the above-identified application.

The last known address for Changning Yan is:

1800 El Paseo, #306
Houston, Texas 77054

It is believed that a fee of **$200.00** is due under 37 C.F.R. § 1.17(g) for submission of this Petition. Accordingly, please charge the requisite fee to Jones Day Deposit Account No.

50-3013. Should any additional fees be required, please charge the required fees to Jones Day Deposit Account No. 50-3013.

Respectfully submitted,

Date: September 29, 2008

Jacqueline Benn 43,492

Jacqueline Benn (Reg. No.)

JONES DAY
222 East 41st Street
New York, New York 10017-6702
(212) 326-3939

E-File

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Fung et al.	Confirmation No.: 4825
Serial No.: 10/583,927 (National Stage of Int'l Application No. PCT/US04/43501)	Art Unit: To be assigned
Int'l Filing Date: December 23, 2004	Examiner: To be assigned
For: NOVEL ANTI-IL-13 ANTIBODIES AND USES THEREOF	Attorney Docket No.: 12279-187-999

DECLARATION OF HONG-VAN M. LE
IN SUPPORT OF PETITION SUBMITTED UNDER 37 C.F.R. § 1.47(a)

Mail Stop PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Hong-Van M. Le, hereby declare that:

1. I am an Intellectual Property Legal Intern at the law offices of Jones Day.

2. I make this Declaration in support of the Petition under 37 C.F.R. §1.47(a) and Response to Notification of Defective Response ("Notice") submitted concurrently herewith for the above-identified patent application. The Notice mailed August 29, 2008 stated that the current declaration does not comply with 37 C.F.R. § 1.497(a) and (b) in that the first and third inventors' first names are different compared to the published International Application. A new declaration listing the names of all the inventors was prepared and sent to the inventors for their signatures. Despite diligent efforts, joint inventor Changning Yan cannot be reached to execute a declaration in connection with the above-identified patent application. Pursuant to 37 C.F.R. § 1.47(a), I hereby submit proof of the pertinent facts,

describing the diligent but unsuccessful efforts to obtain the signature of Changning Yan for the Declaration.

3. The last known address of Changning Yan is 1800 El Paseo, #306, Houston, Texas 77054, based on the declaration executed by Changning Yan on June 24, 2006 for the above-identified application and on information obtained from prior assignee, Tanox, Inc. This information also indicates that the last known e-mail address of Changning Yan is changnin_yan@yahoo.com.

4. On September 9, 2008, I sent an e-mail to the e-mail address changnin_yan@yahoo.com asking Dr. Yan to confirm whether 1800 El Paseo, #306, Houston, TX 77054 was her current address. A print-out of my e-mail dated September 9, 2008 is attached as Exhibit A. I did not receive any system-generated return e-mails indicating that there was a delivery failure. I did not receive a response from Dr. Yan.

5. On September 10, 2008, I sent an e-mail to Mason (Meisheng) Lu, a named inventor of the above-identified application, to inquire about Dr. Yan's contact information. Dr. Lu informed me in his return e-mail, a copy of which is attached as Exhibit B, that Dr. Yan's e-mail address is changnin_yan@yahoo.com, which is the same e-mail address discussed in paragraphs 3 and 4 above, and that he does not have Dr. Yan's current telephone number.

6. On September 12, 2008, I sent an e-mail to the e-mail address changnin_yan@yahoo.com forwarding a Declaration containing the last known address of Changning Yan, and requested that Dr. Yan either sign and date the Declaration if the information for her is correct, or provide the correct information regarding her address and citizenship. I did not receive any system-generated return e-mails indicating that there was a delivery failure. I did not receive a response from Dr. Yan.

7. On September 16, 2008, I sent an e-mail to Sek Chung Fung (Michael S. C. Fung), a named inventor of the above-identified application, to inquire about Dr. Yan's contact information. Dr. Fung informed me in his return e-mail, a copy of which is attached as Exhibit C, that he did not know Dr. Yan's contact information.

8. On September 17, 2008, I sent an e-mail to the e-mail address changnin_yan@yahoo.com, again forwarding a Declaration containing the last known address of Dr. Yan, and requested that Dr. Yan either sign and date the Declaration if the information for her is correct, or provide the correct information regarding her address and citizenship. On September 21, 2008, September 23, 2008, and September 24, 2008, I also sent e-mails to the e-mail address changnin_yan@yahoo.com requesting that Dr. Yan confirm receipt of my previous e-mails. I did not receive any system-generated return e-mails indicating that there was a delivery failure for any of these e-mails. I did not receive a response from Dr. Yan.

9. On September 21, 2008, I paid $7.90 to obtain a search report for the name "Changning Yan" from the internet website http://www.peoplelookup.com. A print-out of the search report sent to my Yahoo e-mail address is attached hereto as Exhibit D. The search report listed 3 addresses and two different telephone numbers, *i.e.*, 713-790-9627 and 409-763-2895. There are no dates associated with the addresses and telephone numbers, thus I cannot determine which address and/or telephone number is current. I have dialed the telephone number 713-790-9627 several times, and each time, I was informed by an operator recording that the number has been disconnected or is no longer in service. I have dialed the telephone number 409-763-2895 approximately 3 times on different days, and each time, there is no ring tone. Thus, I am not able to verify whether any of the addresses and telephone numbers from the search report are those of Dr. Yan currently.

10. An internet search conducted by me at the web site http://www.google.com with the search terms "Changning Yan" revealed that Yan *et al.*, Biol. Reprod. 2006 Jun;74(6):999-1006 is a recent publication, of which Changning Yan is an author. A print-out of the first page of results obtained from the internet search is attached hereto as Exhibit E, and a copy of the Yan et al. publication is attached hereto as Exhibit F. The Yan et al. publication provided an e-mail address for a co-author, Dr. Martin M. Matzuk (mmatzuk@bcm.tmc.edu). On September 22, 2008, I sent an e-mail to Dr. Matzuk to inquire about Dr. Yan's contact information. Dr. Matzuk informed me in his return e-mail, a copy of which is attached as Exhibit G, that he last heard that Dr. Yan was still living and working in Houston, but he did not provide a contact information.

11. On September 23, 2008, I sent an e-mail to Dan Huang, a named inventor of the above-identified application, to inquire about Dr. Yan's contact information. Dr. Huang informed me in his return e-mail, a copy of which is attached as Exhibit H, that he did not have Dr. Yan's contact information.

12. On September 23, 2008, I sent a letter forwarding a Declaration for execution to Dr. Changning Yan at 1800 El Paseo, #306, Houston, Texas 77054 via UPS shipping for next day air delivery. The UPS tracking number 1Z10445E2595723443 was assigned to the shipment. A copy of the letter and the UPS shipping label bearing the tracking number 1Z10445E2595723443 are attached hereto as Exhibit I. A tracking summary retrieved on September 26, 2008 using the UPS tracking system on the internet at the website http://www.ups.com/content/us/en/index.jsx indicated that the letter was delivered on September 24, 2008 at 10:04 a.m., and it was signed for by "Hernandez." A print-out of the tracking summary is attached as Exhibit J. I have not received any responses or communications from Dr. Yan.

13. On September 25, 2008, I sent an e-mail to Matthew Moyle, a named inventor of the above-identified application, to inquire about Dr. Yan's contact information. Dr. Moyle informed me in his return e-mail, a copy of which is attached as Exhibit K, that he did not have Dr. Yan's contact information.

14. Thus, I am unable to reach Changning Yan.

15. I declare further that all statements made in this Declaration of my knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: September 29, 2008

Hong-Van M. Le

EXHIBIT A

Hong-Van M Le/JonesDay
Extension 7-3786
09/09/2008 02:59 PM

To changnin_yan@yahoo.com
cc Jacqueline Benn/JonesDay@JonesDay
bcc
Subject U.S. Patent Application No. 10/583,927

U.S. Patent Application No. 10/583,927
(National Stage of PCT/US2004/043501)
Entitled: Novel Anti-IL-13 Antibodies and Uses Thereof
International Filing Date: December 23, 2004
Inventors: Fung et al.

Dear Dr. Yan:

You are a named inventor of the above-identified application together with Dr. Sek Chung Fung, Dr. Mason Lu, Dr. Matthew Moyle, Dr. Sanjaya Singh, and Dr. Dan Huang.

We must prepare and file an executed Declaration of the Inventors to meet the statutory requirements of the United States Patent and Trademark Office. The Declaration of the Inventors contains the inventors' addresses and citizenship and requires the inventors' signatures. Thus, we ask for your assistance in this matter. In particular, please confirm whether the following is your current address:

1800 El Paseo, #306
Houston, TX 77054

If the above address is incorrect, we would appreciate it if you could provide us with your current address so that we can prepare a Declaration of the Inventors for your signature. Also, please confirm your citizenship. Our records indicate that you are a citizen of the People's Republic of China.

The **non-extendible** due date for filing the Declaration of the Inventors is **September 29, 2008**. We look forward to hearing from you soon.

If you have any questions, please do not hesitate to contact us. Thank you for your assistance in this matter.

Best regards,

Van

Hong-Van M. Le, Ph.D.
Intellectual Property Legal Intern
Jones Day
222 East 41st Street
New York, NY 10017-6702
Tel: 1-212-326-3786
Fax: 1-212-755-7306

EXHIBIT B

"ML" <mmjj78@gmail.com>

09/11/2008 12:06 AM

To "'Hong-Van M Le'" <hmle@JonesDay.com>
cc <changnin_yan@yahoo.com>
bcc
Subject RE: U.S. Patent Application No. 10/583,927

History: This message has been replied to.

Dear Van,

I have got Ms. ChangNin Yan's email address from my previous colleague Dr. Zou, but he doesn't have her current phone number.

ChangNin's email is changnin_yan@yahoo.com and I send this email as a copy to her as well.

Would you please let me know if you need any more help?

Thanks.

Best regards,

Mason

Mason Lu, MD., PhD.
MabStar, Inc.
2260 W. Holcombe Blvd., Suite 406
Houston, Texas 77030
Tel: 713-669-0511;
Mobile1: 832-866-0458 (Houston)
Mobile2: 158-0062-2355 (Shanghai);
Fax: 713-669-0076;
Email1: mmjj78@gmail.com
Email2: mlu@mabstar.com

From: Hong-Van M Le [mailto:hmle@JonesDay.com]
Sent: Wednesday, September 10, 2008 1:51 PM
To: ML
Subject: RE: U.S. Patent Application No. 10/583,927

Dear Mason:

I am also trying to contact Dr. Changning Yan regarding the Declaration of the Inventors as well, but I haven't had much success. If you have an e-mail address or phone number for Dr. Yan, could you provide me with that information? Thanks so much.

Best regards,

Van

Hong-Van M. Le, Ph.D.
Intellectual Property Legal Intern
Jones Day
222 East 41st Street
New York, NY 10017-6702
Tel: 1-212-326-3786
Fax: 1-212-755-7306

EXHIBIT C

SEK FUNG
<fungsc@verizon.net>

09/16/2008 02:54 PM

Please respond to
fungsc@verizon.net

To Hong-Van M Le <hmle@JonesDay.com>

cc

bcc

Subject Re: Declaration of the Inventors - Genentech Ref.: P4096R1-US; JD Ref.: 12279-187-999

History: **This message has been replied to.**

Dear Van:

Unfortunately I do not know her contact information since she left Tanox. I understand from Mason Lu (a co-inventor on the patent) that he is trying to contact another former colleague for her contact information. I wonder whether Mason has been able to help you. Thank you.

Have a nice day!

Michael

--- On **Tue, 9/16/08, Hong-Van M Le** ***<hmle@JonesDay.com>*** wrote:
From: Hong-Van M Le <hmle@JonesDay.com>
Subject: Re: Declaration of the Inventors - Genentech Ref.: P4096R1-US; JD Ref.: 12279-187-999
To: "SEK FUNG" <fungsc@verizon.net>
Date: Tuesday, September 16, 2008, 2:41 PM

Dear Michael:

I am sorry to bother you about this, but I have not been able to contact Dr. Changning Yan. I sent several e-mails to changnin_yan@yahoo.com, but I have not received a response yet. If you know of a number or address where I can contact Dr. Yan, I would appreciate it if you could let me know. Thank you.

Best regards,

Van

Hong-Van M. Le, Ph.D.
Intellectual Property Legal Intern
Jones Day
222 East 41st Street
New York, NY 10017-6702
Tel: 1-212-326-3786
Fax: 1-212-755-7306

EXHIBIT D

YAHOO! MAIL Classic

People Lookup Report Sunday, September 21, 2008 1:48 PM

From: "PeopleLookup" <reports@peoplelookup.com>
To: hvle1978@yahoo.com

hvle1978@yahoo.com [Sign Out] | M

People Lookup

" I lost touch with my Marine friends years ago but found them again on your site last month. Last week I flew down and stayed with

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PEOPLE LOOKUP REPORT - SEPTEMBER 21, 2008

P YOUR SEARCH:

NAME Changning Yan

REPORT CONTENTS:

- People Search Report

Run Another People Lookup Report

People Search Report

	Name	Age	Birth Date	Address / Phone	Previous Cities
1	**Changning M Yan** **RELATIVES:** Cheng M Yan Chang M Yan C M Yan Chang-ming Yan Qing Yan Chunhong Yan Jiong Yan	40	**DOB:** 06/08/1968	**Address 1 Confirmed:** 8282 CAMBRIDGE #2306 HOUSTON, TX 77054 (713) 790-9627 Connected **Address 2:** 1608 MARKET ST GALVESTON, TX 77550 (409) 763-2895 **Address 3:** 612 12TH GALVESTON, TX 77550 (409) 763-2895	Houston, TX Galveston, TX Pearland, TX Humble, TX Waco, TX See all addresses

What is a P

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EXHIBIT E

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en.scientificcommons.org/**changning_yan** - 10k - Cached - Similar pages

Yan, C (**Changning**)
Yan, C (**Changning**). Latest papers:. Biol Reprod. 2006 Feb 22; : 16495478 (P,S,E, B,D). Regulation of Growth Differentiation Factor 9 Expression in Oocytes In ...
lib.bioinfo.pl/auid:1612801 - 42k - Cached - Similar pages

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Molecular Endocrinology -- Table of Contents (18 [6])
Xuemei Wu, Lei Chen, Christopher A. Brown, **Changning Yan**, and Martin M. Matzuk: Interrelationship of Growth Differentiation Factor 9 and Inhibin in Early ...
mend.endojournals.org/content/vol18/issue6/ - Similar pages

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EXHIBIT F

BIOLOGY OF REPRODUCTION **74**, 999–1006 (2006)
Published online before print 22 February 2006.
DOI 10.1095/biolreprod.105.050013

Regulation of Growth Differentiation Factor 9 Expression in Oocytes In Vivo: A Key Role of the E-Box[1]

Changning Yan,[3] Julia A. Elvin,[3,4] Yi-Nan Lin,[3,5] Lou Ann Hadsell,[5] Jie Wang,[5] Francesco J. DeMayo,[5] and Martin M. Matzuk[2,3,4,5]

Departments of Pathology,[3] Molecular and Human Genetics,[4] and Molecular and Cellular Biology,[5] Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

Growth differentiation factor 9 (*GDF9*) is preferentially expressed in oocytes and is essential for female fertility. To identify regulatory elements that confer high-level expression of *GDF9* in the ovary but repression in other tissues, we generated transgenic mice in which regions of the *Gdf9* locus were fused to reporter genes. Two transgenes (−10.7/+5.6m*Gdf9-GFP*) and (−3.3/+5.6m*Gdf9-GFP*) that contained sequences either 10.7 or 3.3 kb upstream and 5.6 kb downstream of the *Gdf9* initiation codon demonstrated expression specifically in oocytes, thereby mimicking endogenous *Gdf9* expression. In contrast, transgenes −10.7m*Gdf9-Luc* and −3.3m*Gdf9-Luc*, which lacked the downstream 5.6-kb region, demonstrated reporter expression not only in oocytes but also high expression in male germ cells. This suggests that the downstream 5.6-kb sequence contains a testis-specific repressor element and that 3.3 kb of 5′-flanking sequence contains all the *cis*-acting elements for directing high expression of *Gdf9* to female (and male) germ cells. To define sequences responsible for oocyte expression of *Gdf9*, we analyzed sequences of *Gdf9* genes from 16 mammalian species. The approximately 400 proximal base pairs upstream of these *Gdf9* genes are highly conserved and contain a perfectly conserved E-box (CAGCTG) sequence. When this 400-bp region was placed upstream of a luciferase reporter (−0.4m*Gdf9-Luc*), oocyte-specific expression was observed. However, a similar transgene construct (−0.4MUT-m*Gdf9-Luc*) with a mutation in the E-box abolished oocyte expression. Likewise, the presence of an E-box mutation in a longer construct (−3.3MUT-m*Gdf9-Luc*) abolished expression in the ovary but not in the testis. These observations indicate that the E-box is a key regulatory sequence for *Gdf9* expression in the ovary.

evolutionary conservation, gene regulation, oocyte-specific

INTRODUCTION

The critical importance of the oocyte-secreted protein growth differentiation factor 9 (GDF9) in ovarian function has been established by both in vivo and in vitro studies. Female mice lacking GDF9 are infertile because of a block at the primary follicle stage in the ovary, but *Gdf9* knockout males are normal [1]. Furthermore, our laboratory [2–4] and others [5–8] have shown that recombinant GDF9 enhances granulosa cell growth during folliculogenesis and regulates key factors and proteins (e.g., kit ligand, cyclooxygenase 2, hyaluranon synthase 2, LH receptor, urokinase plasminogen activator, pentraxin 3, prostaglandins, and progesterone) in early folliculogenesis and the periovulatory period. Thus, appropriate expression of *GDF9* is critical for maintaining normal ovarian functions in vivo.

The *Gdf9* mRNA and GDF9 protein are highly expressed in oocytes of the ovary [2, 9, 10]. *Gdf9* was thought to be expressed exclusively in the ovary, until it was found to be expressed at much lower levels in the testis and hypothalamus as well [11]. The function of this low-level expression of *Gdf9* in these other tissues is unclear, because *Gdf9* knockout males are fertile and show no gross physical or behavioral defects [1]. In the mouse ovary, *Gdf9* mRNA and its protein are not expressed in primordial (quiescent) follicles (types 1 and 2) but are expressed beginning at the early primary follicle (type 3a) stage through ovulation. Among the few identified oocyte-specific genes, *Gdf9* shares an identical expression pattern in the ovary with the zona pellucida (*Zp*) genes. Mouse *Gdf9* and mouse *Zp* mRNA are expressed in type 3a follicles and at high levels in larger growing oocytes [10, 12]. In both *Zp3* and *Gdf9* promoters, an E-box consensus sequence (CANNTG) is present [13]. The E-box sequences have been identified in several tissue-specific genes and shown to be involved in tissue-specific expression by binding heterodimers of helix-loop-helix transcription factors [14–17]. An in vitro study of the mouse *Zp3* promoter identified an E-box (CACGTG) consensus sequence at 186 bp upstream of the transcription start site and showed that the E-box was necessary and sufficient for directing luciferase expression in microinjected oocytes [13]. In the studies herein, alignment of the proximal promoters of the mouse, rat, dog, sheep, chimpanzee, and human *GDF9* genes revealed a perfectly conserved E-box sequence (CAGCTG). Given the similarity of the gene expression patterns in the ovary of *Gdf9* and *Zp* genes and the similar E-box locations in the promoters of mouse *Zp3* and *Gdf9* genes, we hypothesized that the conserved E-box may play a role in the oocyte-specific expression of *Gdf9* in the ovary.

Oocyte-expressed genes are difficult to study in vitro for several reasons. As a result of the unavailability of oocyte cell lines (because oocytes do not undergo mitosis), simple transfection into a cell line is not an option for studying oocyte-expressed genes in vitro. Additionally, the protective zona pellucida of freshly harvested oocytes prevents utilization of standard transfection modalities (e.g., lipofection and electroporation). Oocyte microinjection is an alternative for the transient expression of oocyte genes and has been used successfully [13]. However, because of the difficulty of maintaining the viability of oocytes in vitro and the limitation of the in vitro experiments performed in these single cells, such

[1]Supported in part by National Institutes of Health (NIH) grant HD33438.
[2]Correspondence: Martin M. Matzuk, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. FAX: 713 798 5833; e-mail: mmatzuk@bcm.tmc.edu

Received: 3 December 2005.
First decision: 25 December 2005.
Accepted: 22 February 2006.

ISSN: 0006-3363. http://www.biolreprod.org

studies cannot address whether the gene expression is oocyte-specific (i.e., cannot define the sequences that restrict expression to the ovary versus other tissues). Therefore, we decided to pursue a transgenic approach. Although the generation of transgenic animals requires a significantly longer time, the transgene approach defines a broader scope of the expression at intraovarian and extraovarian sites. Here, we report successful use of the transgene strategy to study systematically the promoter regions of mouse *Gdf9* in vivo.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise indicated, all chemicals and reagents were obtained from Sigma and Fisher. Unless otherwise specified, restriction enzymes were obtained from New England Biolabs.

Construction of Gdf9 Promoter-Reporter Gene Constructs

A full 16.3 kb of m*Gdf9* genomic sequence from a phage clone, including 10.7 kb of 5′-flanking region of m*Gdf9* and approximately 5.6 kb downstream of the transcription site of the gene, including exon 1 (397 bp), intron 1 (2.8 kb), exon 2 (1285 bp), and approximately 1 kb of the 3′ downstream region, was subcloned into pBluescript SK vector (pB/S4). The initiation codon ATG of m*Gdf9* in pB/S4 was altered by site-directed mutagenesis into a *Bam*HI site (pE22). The green fluorescent protein (GFP) plasmid was a kind gift from Dr. Roger Tsien (Department of Pharmacology, Department of Chemistry and Biochemistry, and Howard Hughes Medical Institute, University of California at San Diego). To generate the transgene −10.7/+5.6m*Gdf9-GFP*, 714 bp of the coding region of GFP without its own polyadenylation site were inserted into the *Bam*HI site of the pE22. With the convenient restriction enzyme digestion, sequences between −10.7 and −3.3 kb of the transgene −10.7/+5.6m*Gdf9-GFP* were deleted and religated to generate new transgene, −3.3/+5.6m*Gdf9-GFP*. Transgene −3.3/+5.6m*Gdf9-GFP* contained 3.3 kb of 5′-flanking region of m*Gdf9* and approximately 5.6 kb downstream sequence linked to an enhanced version of GFP.

For m*Gdf9* promoter-luciferase constructs, pGL3-basic (Promega) containing firefly luciferase coding sequence was modified by inserting an oligonucleotide linker into its multiple cloning sites to generate appropriate restriction enzyme sites for subcloning. The 3.3 and 0.4 kb from the 5′-flanking region of m*Gdf9*, with or without the E-box mutation, were products of convenient enzyme digestions of the existing subclones of m*Gdf9*. The resulting different 5′-flanking sequences were ligated into appropriate restriction enzyme sites of the modified pGL3 vector.

The E-box mutation was generated by PCR-based, site-directed mutagenesis. Briefly, oligonucleotides containing the desired sequence changes were synthesized and utilized as primers in PCR. The synthesized oligonucleotides were as follows: OligoCY7, 5′-CTAGCCTCGAGCTGCAGATCTA-3′, and oligoCY8, 5′-AGCTTAGATCTGCAGCTCGAGG-3′. The E-box consensus sequence CAGCTG was changed into an *Xba*I-site TCTAGA. Plasmid pE5, which contains 5′-flanking region of the *Gdf9*, was used as a template. Two separate PCRs were performed with primer pairs OligoCY7/T7 and OligoCY8/T3, respectively. The PCR was performed with Pfu DNA polymerase (Stratagene) under the PCR conditions described by the manufacturer. The purified PCR products from the two separated PCRs were mixed, denatured at 94°C, and then cooled to room temperature for annealing. The resulting product was used as template in another round of PCR with primers T7/T3. The PCR products were purified, digested with *Pst*I and *Bam*HI, and then cloned into the same sites of pBluescript SK vector (Stratagene). The DNA sequencing analysis was utilized to confirm the mutation.

Generation of Transgenic Mice

Linearized transgene fragments were purified, quantitated, and microinjected into the pronucleus of fertilized eggs of C57BL/6/C3H × ICR hybrids [2]. Microinjected eggs were then transferred into oviducts of pseudopregnant foster mothers. Transgenic mice were identified by Southern blot analysis or PCR. Briefly, mouse tail genomic DNA was isolated by overnight digestion with proteinase K followed by ethanol precipitation. The DNA was then dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and subjected to overnight restriction enzyme digestion. The digestion was electrophoresed on 0.7% agarose gel and transferred to nylon membranes (Amersham). Probes were generated by random priming kit (Pharmacia) and hybridized with the blots. The [^{32}P]GFP probe and enhanced [^{32}P]EGFP probe were used, respectively, for detecting GFP transgenic and EGFP transgenic mice.

A primer pair, one from luciferase sequence 5′-CTAGCCTCGAGCTGCAGATCTA-3′ and the other from the 5′ sequence of m*Gdf9*-5′-AGCTTAGATCTGCAGCTCGAGG-3′, was synthesized for PCR genotyping. A small portion of tail genomic DNA was used as the PCR template. The PCR was performed with Hot-Start Taq DNA polymerase (Qiagen) under the conditions described by the manufacturer. The resulting PCR products were electrophoresed on ethidium bromide/agarose gel and visualized under ultraviolet (UV) light.

To confirm the presence of the E-box mutation in the transgenics, the PCR product was further digested with *Xba*I, electrophoresed on an ethidium bromide/agarose gel, and visualized under UV light.

All mice used in these studies were generated and maintained at Baylor College of Medicine. Mice were kept under standard laboratory conditions and maintained as per the National Institutes of Health guidelines and approved animal protocols of the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Multitissue Northern Blot Analysis

Total RNA was extracted from the tissues of the transgene-positive mice using RNA STAT-60 according to the manufacturer's instruction (Leedo Medical Labortories). Poly(A)$^+$ mRNA was prepared by mRNA purification kit (Stratagene). Fifteen micrograms of total RNA or 10 μg of mRNA were electrophoresed on 1.2% denaturing agarose gel, transferred to a Hybond-N nylon membrane (Amersham), and cross-linked by UV irradiation. Probes were generated using a Strip-EZ DNA probe synthesis and removal kit (Ambion). The RNA membrane was first hybridized with the [^{32}P]GFP or [^{32}P]EGFP coding region, then stripped and reprobed with 18S for the RNA loading control.

In Situ Hybridization

In situ hybridization was performed as described previously [18]. Freshly dissected ovaries from transgenic mice were fixed in 4% paraformaldehyde overnight. The ovaries were incubated sequentially in the following solutions: 1× PBS, 0.85% NaCl, 1:1 ethanol:saline, and 70% ethanol. Testes from transgenic mice were fixed in Bouin fixative at room temperature for 3 h. The testes were incubated in the multiple changes of 70% ethanol. The fixed ovaries and testes were then embedded in paraffin. Five-μm sections were cut, processed, and pretreated as described. The [α-^{35}S-UTP]GFP or [α-^{35}S-UTP]EGFP antisense and sense probes were generated using T7 or T3 Riboprobe Systems (Promega). Hybridizations were carried out at 55°C for 12–16 h with 5×10^6 cpm of each riboprobe per slide in a solution containing 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM $NaPO_4$ (pH 8.0), 10% dextran sulfate, 1× Denhart solution, and 0.5 mg/ml of yeast tRNA. Slides were washed sequentially at 55°C in 5× SSC (1×: 0.15 M sodium chloride and 0.015 M sodium citrate) and 10 mM β-mercaptoethanol and then in 50% formamide, 2× SSC, and 10 mM β-mercaptoethanol; then, slides were treated with 20 μg/ml of RNase A in 1× TEN (10 mM Tris-HCl, 5 mM EDTA, 500 mM NaCl) buffer for 30 min at 37°C. A high-stringency wash was carried out at 65°C in a solution containing 2× SSC, 50% formamide, and 50 mM β-mercaptoethanol for 20 min; in 2× SSC for 15 min; and then in 0.1× SSC for 10 min. Slides were then dehydrated and subjected to autoradiography with NTB-2 emulsion (Eastman Kodak). After developing and fixing, the slides were counterstained with hematoxylin and mounted for photography.

Luciferase Activity Assay

Tissues collected from transgene-positive and -negative mice were homogenized in 1× lysis buffer (Promega) with a Tissue Tearor electronic homogenizer (Biospec Products). The homogenate was centrifuged at 20 000 × *g* for 5 min at 4°C, and the supernatant was analyzed for luciferase activity by a Luciferase Assay kit as described by the manufacturer (Promega). Luciferase activity was measured with a luminometer. The luciferase final activity was normalized by the protein level in the tissues. Total protein was measured by the BCA protein assay kit (Pierce).

RESULTS

16.3 kb of Gdf9 Flanking Sequences Recapitulate Endogenous Gdf9 Expression

To maximize the likelihood of including all *cis*-acting elements necessary for oocyte-specific expression of *Gdf9*, our

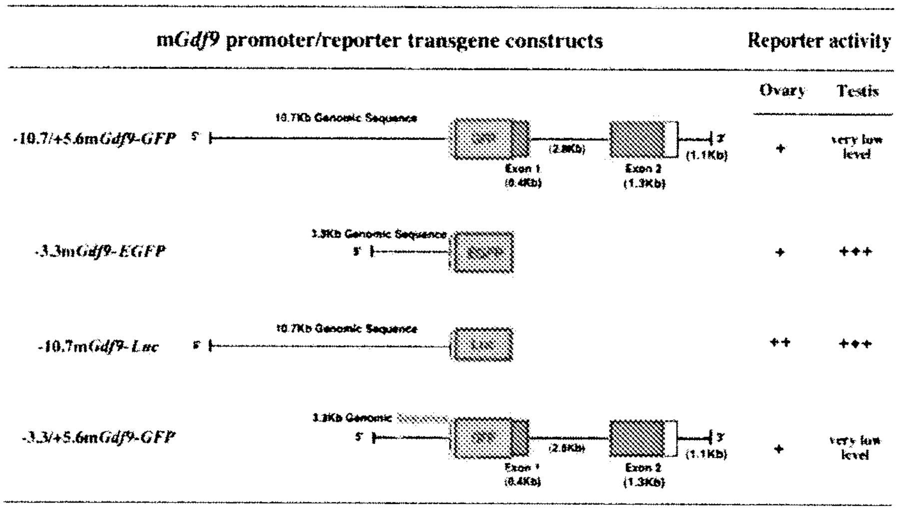

FIG. 1. Expression of four reporter transgenes with or without the 5.6 kb downstream region in ovary and testis. The relative strength of reporter activity was described as low (+), medium (++), or high (+++).

initial transgene (−10.7/+5.6m*Gdf9-GFP*) (Fig. 1) contained all the genomic sequences between −10.7 and +5.6 kb (10.7 kb upstream and 5.6 kb downstream from the initiation codon of mouse *Gdf9*) [19]. Two independent transgenic lines expressing *GFP* mRNA were obtained. Northern blot analysis showed that the *GFP* reporter gene was expressed in the ovary and only minimally in the other tissues examined (Fig. 2A). Multiple transcript sizes are observed in the −10.7/+5.6m*Gdf9-GFP* mice. These transcripts likely result from alternative polyadenylation sites, as observed in other testis and ovary transcripts [20, 21]. The *GFP* expression was only detectable in testis and brain when poly(A)$^+$ RNA from these two transgenic lines were used (Fig. 2A). The *GFP* signal was further localized to the oocytes of the ovary by in situ hybridization analysis (Fig. 3, A and B). Thus, the 16.3 kb of genomic sequence flanking the *Gdf9* gene contains all the elements required for efficient expression in oocytes and relative suppression of expression elsewhere. Identical to mouse *Gdf9* expression in the ovary [2, 9, 10], the expression of transgene −10.7/+5.6m*Gdf9-GFP* was not present in primordial oocytes, was first detected in oocytes of type 3a follicles, and was present in oocytes of all subsequent stages.

Compared to −10.7/+5.6m*Gdf9-GFP*, the transgene −3.3/+5.6m*Gdf9-GFP* contained the same sequences from −3.3 to +5.6 kb, but with a deletion of upstream sequences between −10.7 and −3.3 kb (Fig. 1). Four founder lines were transgene-positive, as demonstrated by Southern blot analysis, and three lines transmitted the transgene. Similar to the expression pattern of the 16.3-kb transgene, the expression of the 8.9-kb transgene was restricted to the oocytes of the ovary, as demonstrated by Northern blot analysis (Fig. 2, C and D) and in situ hybridization analysis (Fig. 3, C and D). Variable expression of the 8.9-kb transgene was seen in testis samples from males of these lines (Fig. 2, C and D). Higher expression of the 8.9-kb transgene was detected in the brain samples of multiple lines, although the size of the transcript was aberrantly large (Fig. 2, C and D). This suggests that these brain transcripts likely were derived from expression of other genes located 5′ of the *Gdf9* locus, possibly spliced to the *GFP* reporter coding region. When looking into the upstream region, an ubiquinol-cytochrome *c* reductase-binding protein gene (UniGene Mm.251621) was found approximately 2.5 kb upstream from *Gdf9*. Because this gene is expressed in multiple tissues and is transcribed in the opposite direction from *Gdf9*, it is unlikely to be responsible for the longer brain

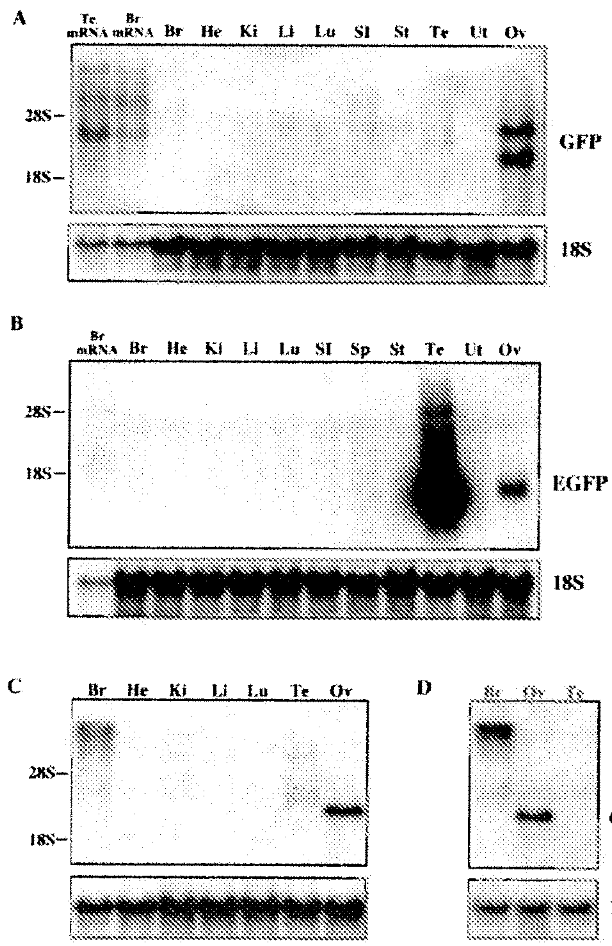

FIG. 2. Multitissue Northern blot analysis of transgene expression. The expression levels from transgene −10.7/+5.6m*Gdf9-GFP* (**A**), transgene −3.3m*Gdf9-EGFP* (**B**), and transgene −3.3/+5.6m*Gdf9-GFP* (**C** and **D**) are shown. Fifteen micrograms of total RNA or 10 μg of Poly(A)$^+$ mRNA per lane were loaded on gel. The Northern blots were first probed with GFP (**A**, **C**, and **D**; top) or EGFP (**B**; top) and then stripped and reprobed with 18S cDNA as loading control (bottom). The data shown here are representative of two transgenic lines from transgene −10.7/+5.6m*Gdf9-GFP*, two transgenic lines from transgene −3.3m*Gdf9-EGFP*, and three transgenic lines from transgene −3.3/+5.6m*Gdf9-GFP*. Br, Brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Ov, ovary; Sp, spleen; St, stomach; SI, small intestine; Te, testis.

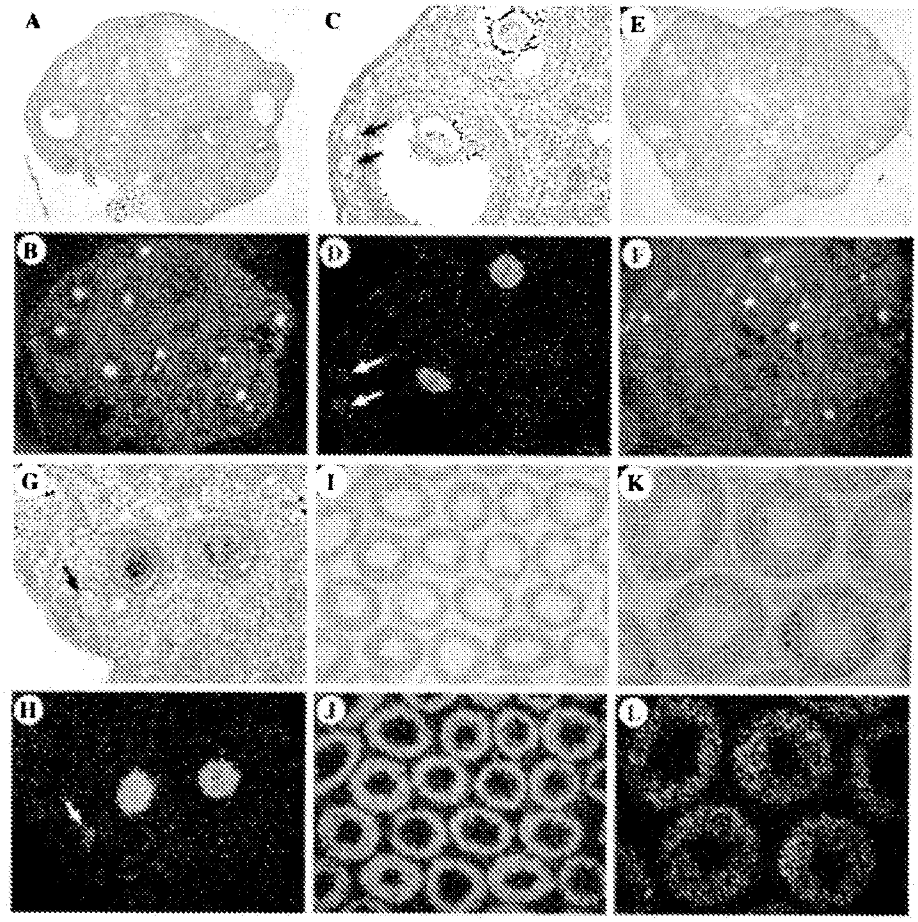

FIG. 3. In situ hybridization analysis of transgene expression in ovary and testis. Ovary sections from transgene −10.7/+5.6m*Gdf9-GFP* (**A** and **B**), transgene −3.3/+5.6m*Gdf9-GFP* (**C** and **D**), and transgene −3.3m*Gdf9-EGFP* (**E–H**) showed that *GFP* or *EGFP* was expressed exclusively in the oocytes of the ovary. Arrows (**I–L**) indicate the detectable levels of reporter transcripts in the oocytes of primary follicles. Testis sections from transgene −3.3m*Gdf9-EGFP* showed that the RNA of *EGFP* was localized in the round spermatids and the spermatocytes. Pictures were taken under bright-field (**A**, **C**, **E**, **G**, **I**, and **K**) and under dark-field (**B**, **D**, **F**, **H**, **J**, and **L**). Original magnification **A**, **B**, **E**, **F**, **I**, **J** ×50; **K**, **L** ×100; **C**, **D** ×200; **G**, **H** ×400.

transcript. However, one seemingly aberrant cDNA (GenBank accession no. AK078302) from an olfactory brain library is transcribed toward *Gdf9*, showing partial overlap with that ubiquinol-cytochrome *c* reductase-binding protein gene. It is likely that putting the transgene in a different genomic context may have enhanced transcription, extending into the *GFP:Gdf9* locus, similar to that of the aberrant cDNA. Thus, these two constructs showed that sequences between −3.3 and +5.6 kb contain *cis*-acting elements that are sufficient both for directing *Gdf9* expression specifically to oocytes in vivo and for suppressing the expression of *Gdf9* in testis and other tissues.

*3.3 kb of 5′-Flanking Region of m*Gdf9 *Is Sufficient to Drive Expression Specifically in Germ Cells*

We produced an additional transgene (−3.3m*Gdf9-EGFP*) that contained only 3.3 kb of the 5′-flanking sequences of *Gdf9* fused to the cDNA of EGFP (*EGFP*) but that lacked the +5.6 kb downstream sequences of mouse *Gdf9* (i.e., the exons, intron, and some 3′ noncoding regions). Among the five founder lines that were generated, only two lines transmitted the −3.3m*Gdf9-EGFP* transgene. As demonstrated by multi-tissue Northern blot analysis, the *EGFP* mRNA was detected not only in the ovary but also at a much higher level in the testis in both lines (Fig. 2B and data not shown). The other positive line also had detectable expression of *EGFP* mRNA in the lung (data not shown), which likely was an artifact of the genome insertion site.

To quantitate and confirm the high expression level in the testis, we took advantage of the high sensitivity and quantitative features of the luciferase reporter gene. The above 3.3-kb sequences were fused to the coding region of the firefly luciferase gene (−3.3m*Gdf9-Luc*) (Fig. 4). The new transgene −3.3m*Gdf9-Luc* in two independent transgenic lines showed luciferase expression in the gonads in an identical expression pattern to that of transgene −3.3m*Gdf9-EGFP* (data not shown). Luciferase activity in the testis of the −3.3m*Gdf9-Luc* transgenic mice was approximately fivefold higher than that in the ovary (Fig. 4).

To examine the cell populations expressing the reporters in the ovary and testis, we performed in situ hybridization. In the ovary, the signal of *EGFP* mRNA was only detected in the oocytes of the ovary (Fig. 3, E–H). In the testis, the expression was restricted to germ cells and, specifically, primary spermatocytes through round spermatid stages (Fig. 3, I–L). Thus, the upstream 3.3-kb genomic sequences of mouse *Gdf9* are sufficient to drive the expression in both oocytes in the ovary and germ cells in the testis.

To refine the region conferring high expression in the testis, we first tested the distal 5′-flanking region, specifically between −10.7 and −3.3 kb. Similar to transgene −3.3m*Gdf9-Luc*, transgene −10.7m*Gdf9-Luc* was constructed by fusing the entire 10.7 kb of 5′-flanking sequences of *Gdf9* to the cDNA of the luciferase gene. The resulting two transgenic lines were analyzed for the luciferase activity in comparison with the −3.3m*Gdf9-Luc* transgenic line. As shown in Figure 4, in both ovary and testis, the luciferase activity was significantly

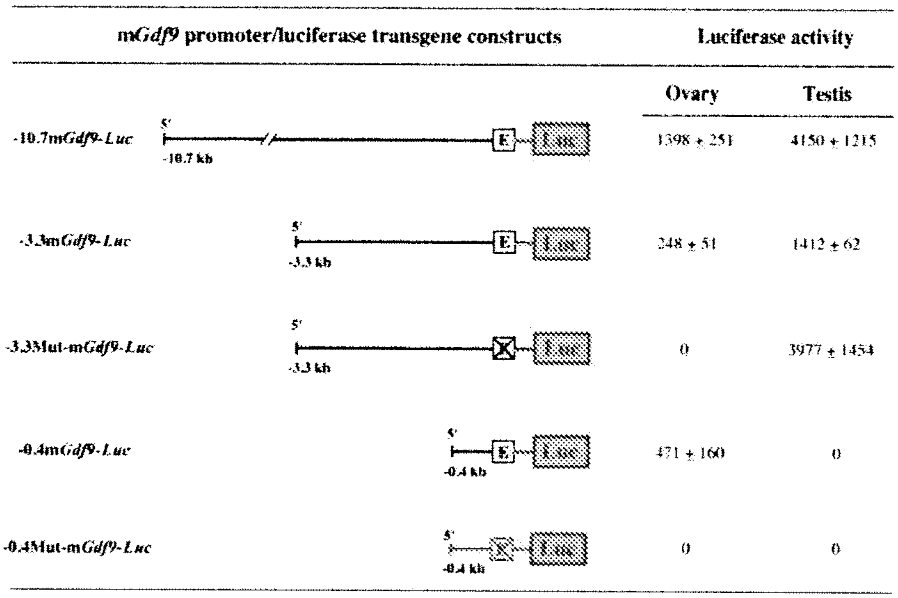

FIG. 4. Expression of five luciferase transgenes with or without the conserved E-box in ovary and testis. The expression levels of −10.7m*Gdf9-Luc* transgene are significantly higher than those of −3.3m*Gdf9-Luc* transgene in ovary ($P < 0.05$) and testis ($P < 0.1$).

higher in the −10.7m*Gdf9-Luc* transgenic than in the −3.3m*Gdf9-Luc* transgenic line. Thus, these results suggested the presence of enhancer activity in the upstream region between −3.3 and −10.7 kb and eliminated the possibility of the presence of repressor activity within the upstream 10.7-kb region.

Testis-Specific Repressor Element Was Located Within the 5.6-kb Sequences Downstream of the Start of Translation

We have shown that transgene −10.7m*Gdf9-Luc*, transgene −3.3m*Gdf9-EGFP*, and transgene −3.3m*Gdf9-Luc*, all lacking the +5.6-kb sequences, were capable of driving high-level reporter expressions in testis (Figs. 1, 2, and 4). In contrast, we have demonstrated that the transgenes carrying the 5.6-kb sequences, −10.7/+5.6m*Gdf9-GFP* and −3.3/+5.6m*Gdf9-GFP*, specifically directed the reporter expression to the oocytes in the ovary, but not to the testis (Fig. 1). Together, these data indicate that the downstream 5.6-kb sequences of *Gdf9* must contain a testis-specific repressor element.

E-box Is Required for Expression of Gdf9 Only in Ovary, Not in Testis

The studies described above show that the oocyte expression of *Gdf9* requires only the 3.3-kb upstream region. We therefore wished to define further the sequences that contributed to this expression. Alignment of the proximal promoter sequences from *Gdf9* genes of 16 mammalian species revealed that a perfectly conserved E-box element (CAGCTG) is present (Fig. 5A). In the mouse, this E-box lies at −182 bp upstream of the start of translation. Interestingly, the approximately 400-bp region upstream of the start of translation was well conserved between mouse and human compared to the less conserved 3′-untranslated region (UTR) and downstream region (Fig. 5B). The conservation was still evident when the corresponding upstream regions from these 16 mammalian species were aligned and examined (Fig. 5A). We therefore explored the possibility of whether a minimal promoter containing only 0.4 kb of the mouse *Gdf9* 5′-flanking region and including this conserved E-box was sufficient to direct expression to oocytes. We linked the 0.4-kb sequences to the cDNA of the luciferase reporter gene to generate the transgene −0.4m*Gdf9-Luc*. In all three transgenic lines, the luciferase expression in ovary was at levels comparable to those in the −3.3m*Gdf9-Luc* transgenic mice. Additionally, the expression of the luciferase was restricted to the ovary (Fig. 4 and data not shown).

To examine further the role of the conserved E-box in the mouse *Gdf9* promoter, the E-box consensus sequence CAGCTG in the above −0.4m*Gdf9-Luc* transgene was mutated into an *Xba*I site (TCTAGA). The mutation was confirmed by DNA sequencing. Three founder mice carrying the −0.4Mut-m*Gdf9-Luc* transgene were identified by PCR genotyping. The mutation in the transgene was further confirmed by *Xba*I digestion of the PCR product (data not shown). As analyzed by a luciferase assay, mutation of the E-box in these transgenic mice abolished the luciferase expression in the ovary, in contrast to the observation in the −0.4m*Gdf9-Luc* transgenic mice (Fig. 4). However, neither −0.4m*Gdf9-Luc* nor −0.4Mut-m*Gdf9-Luc* transgenics had detectable luciferase activity in testis (Fig. 4) or other examined tissues (data not shown). These results suggest that the 0.4-kb sequences contain promoter activity sufficient for expression in the ovary, but not for expression in the testis or other nonovarian tissues.

To determine further whether the function of the E-box was ovary-specific, we made the same E-box mutation in transgene −3.3m*Gdf9-Luc*, which contains the longer 3.3-kb promoter sequences of *Gdf9* (−3.3Mut-m*Gdf9-Luc*). The E-box mutation (i.e., *Xba*I site) in all four generated transgenic lines was confirmed (data not shown). Three of the four transgenic lines consistently showed no detectable luciferase expression in the ovaries but high expression in the testes, at levels comparable to those in −10.7m*Gdf9-Luc* transgenics (Fig. 4). In fact, one of these transgenic lines expressed the highest luciferase activity in testis among all of the examined transgenic lines in the present study. Only one line showed luciferase expression in both ovary and testis at a level similar to the transgene carrying wild-type E-box (data not shown). One likely explanation for this E-box-independent ovarian aberrant expression is an influence of enhancer sequences at a different chromosomal transgene integration site. Thus, these observations are in line with the findings from the −0.4Mut-m*Gdf9-Luc* transgenics, suggesting that the conserved E-box plays an essential role for the expression in the ovary but is not required for the expression in the testis.

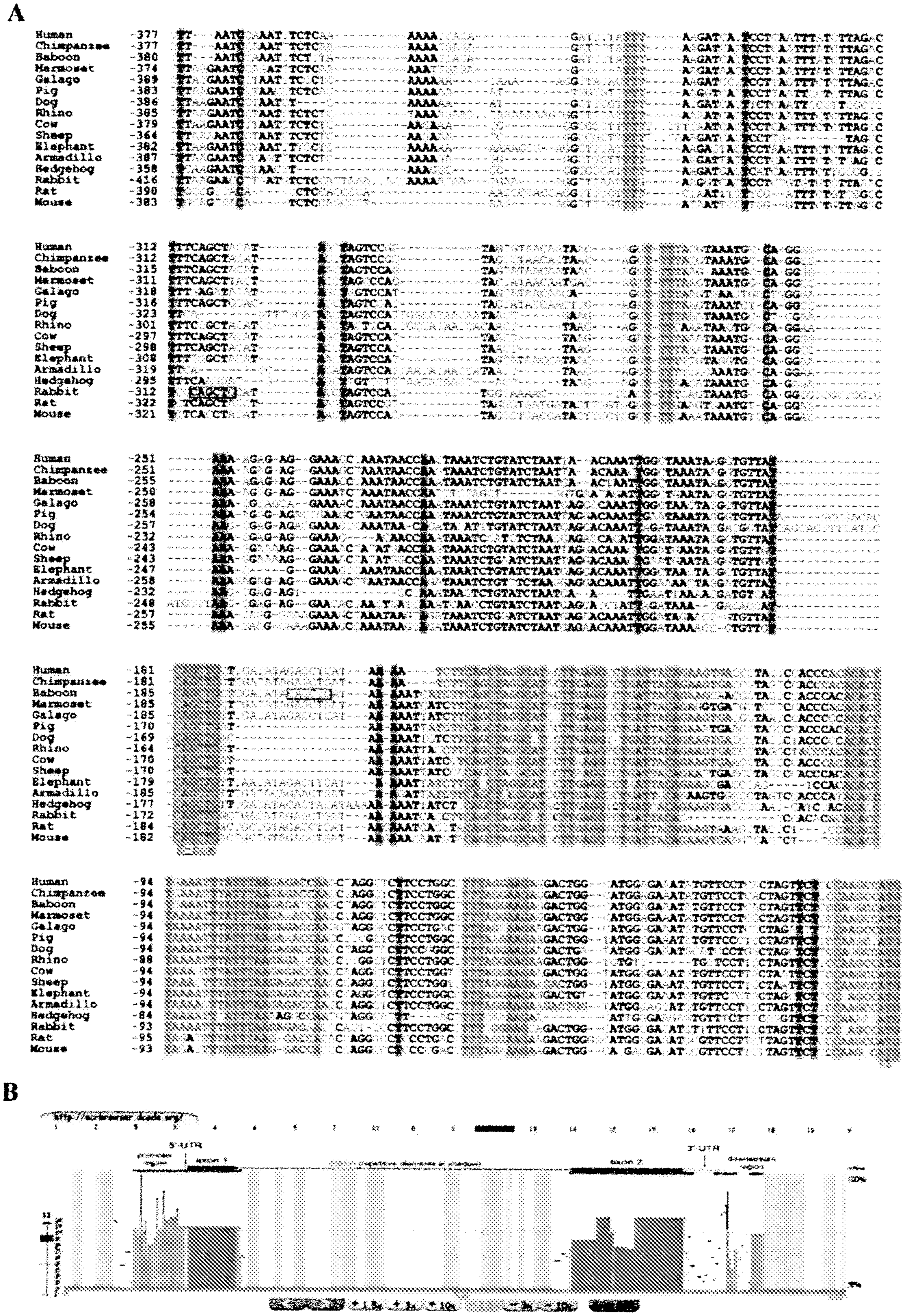

FIG. 5. Phylogenetic footprinting analysis of the approximately 400-bp *Gdf9* proximal promoter region from 16 mammalian species. **A**) Besides some scattered appearances, an E-box (CAGCTG, boxed) is well conserved around −180 relative to the translation start site in the promoter of *Gdf9* across all 16 diverse mammalian species available from various genome projects. Nucleotide sequences conserved in 75% or more of all entries are shown in bold, and those conserved across all entries are further marked with shadow (human, *Homo sapiens*; chimpanzee, *Pan troglodytes*; baboon, *Papio anubis*; marmoset, *Callithrix jacchus*; galago, *Otolemur garnettii*; pig, *Sus scrofa*; dog, *Canis familiaris*; rhino, *Rhinolophus ferrumequinum*; cow, *Bos taurus*; sheep, *Ovis aries*; elephant, *Loxodonta africana*; armadillo, *Dasypus novemcinctus*; hedgehog, *Atelerix albiventris*; rabbit, *Oryctolagus cuniculus*; rat, *Rattus*

DISCUSSION

In the present study, we used transgenic mouse technology to study the promoter of mouse *Gdf9*. Transgenes harboring different regions of *Gdf9* were fused to reporter genes. We demonstrated that transgenes −10.7/+5.6m*Gdf9-GFP*, −3.3/+5.6m*Gdf9-GFP*, and −3.3m*Gdf9-GFP*, were capable of driving the reporter gene expression either specifically to the oocyte or to both oocytes in females and spermatocytes and round spermatids in males. The results suggest that 3.3 kb of the 5′-flanking region of mouse *Gdf9* contains all the *cis*-acting elements that are sufficient for targeting the expression to male and female germ cells.

Comparison of the expression pattern among the transgenes generated in the present study reveals that the 5.6-kb sequences downstream of the initiation codon of mouse *Gdf9* are critical for suppressing *Gdf9* expression in testis. The deletion of this region causes the initiation of high expression in the testis, indicating that a testis-specific repressor element is located within the 5.6-kb region. An unknown testis-specific repressor therefore must interact with the *cis*-acting sequences within this 5.6-kb region to suppress mouse *Gdf9* expression specifically in testis. Many studies have shown that the presence of repressors is one mechanism to control transcription in a tissue-specific manner. This 5.6-kb testis-repressing region of *Gdf9* includes the coding sequences, intron, and 3′-UTR. The 3′-UTR has been demonstrated to function as a repressor binding region in several genes [22, 23]. Regulatory sequences in the 3′-UTR likely are conserved between species [24], and regions showing 50% or more identity were evident when comparing the 3′-UTR and the downstream regions of human *Gdf9* and mouse *Gdf9* (Fig. 5B). Further deletion analysis would help to define these testis-specific repressor elements. A more expedient way to pursue identification of the testis-repressor element would be in vitro transfection into commercially available male germ cell lines. For example, germ cell line-1 (GC-1), derived from testicular germ cells, has been successfully used for in vitro transfection [25]. We cannot detect *Gdf9* mRNA in these GC-1 cells (unpublished data). Thus, this cell line may be useful for further delineating possible repressor elements through an in vitro transfection approach.

An E-box motif (CANNTG) has been found in the promoter of many tissue-specific genes, including the *Zp* genes, *Cyp19*, and P2X1. The consensus sequence CANNTG can bind to proteins of a basic helix-loop-helix (bHLH) family to regulate transcription in a tissue-specific manner. Mutational analysis of the conserved E-box at (−182 to −177 bp) upstream of the *Gdf9* initiation codon has demonstrated that the E-box is essential for the expression of *Gdf9* in the ovary. However, the intact E-box is not required for expression in the testis, suggesting that the E-box functions in an ovary-specific manner. A similar phenomenon has been demonstrated in the mouse *Zp3* gene. An E-box CACGTG present at −210 to −205 bp upstream of the *Zp3* initiation codon is necessary and sufficient for *Zp3* expression in the oocyte, but it is not sufficient for ectopic expression in 10T1/2 cells. Additionally, an ovary-specific bHLH transcription factor, FIG1a , binds to the *Zp3* E-box through heterodimerization with protein E12, a ubiquitous bHLH [13]. The *FIG1a* is expressed at high levels in primordial oocytes and persists in growing oocytes. The expression window of *FIG1a* precedes and is coincident with the *Gdf9* expression. Thus, FIG1a is a likely candidate transcription factor for germ cell-specific regulation of *Gdf9*. However, the E-box (CAGCTG) identified in the *Gdf9* promoter was similar but not identical to the E-box (CACGTG) in *Zp3* gene. It has been observed that the central two nucleotides contribute to discriminatory binding among the different bHLH family members [16, 26]. It will be interesting to determine whether the inversion of the central G and C nucleotides of the E-box permits regulation by FIG1a or requires a novel transcription factor that specifically regulates *Gdf9* expression in the ovary. This question remains to be addressed by further analysis, such as gel mobility shift assays.

In addition to the conserved E-box, several other conserved transcriptional binding sites also were identified by aligning the mammalian *Gdf9* promoter sequences. For example, transcription factor-binding sites conserved in both mouse and human were found by the Match program (http://www.gene-regulation.com/) searching the TRANSFAC 6.0 database. These sites include HNF-3β, FOXD3, GATA-3, GAGA-X, Lmo2 complex, NF-Y, CCAAT box, Pax-4, Brn-2, HLF, and Nkx2–5. The significance of these putative transcription factor-binding sites in regulating *Gdf9* expression remains to be demonstrated.

The 3.3 kb of 5′-flanking region of the mouse *Gdf9* gene has been used to drive Cre recombinase, and the Cre recombinase activity recapitulated *Gdf9* expression in growing oocytes both temporally and spatially [10, 27], allowing the creation of conditional oocyte-specific knockouts beginning from the primordial follicle stage. In addition to *Zp3Cre* [28] and *Msx2Cre* [29], in which the oocyte-specific Cre activity was observed starting from the primary follicle stage and the secondary follicle stage, respectively, *Gdf9-iCre* in mice appears to result in efficient oocyte-specific conditional knockout from the primordial follicle stage. This is particularly important for studying the postnatal functions of germ cell-expressed genes that cause early embryonic lethal or gonad formation failure in conventional knockouts. One candidate for this type of tissue-specific and, in fact, cell type-specific knockout approach is the oocyte-expressed KIT tyrosine receptor. When lost constitutively, absence of KIT leads to defects in embryonic germ cell migration and proliferation, resulting in an absence of germ cells in both the male and female gonad postnatally [30, 31], preventing the study of KIT function during folliculogenesis.

Interestingly, medium to high levels of reporter gene expression were observed in the testis of transgenic mice with all our transgene constructs that utilized the 3.3 kb of 5′-flanking region of the mouse *Gdf9* as promoter, but two of the three transgenic *Gdf9-iCre* lines generated by Lan et al. [27] that utilized the same 5′-flanking region as promoter showed only oocyte-specific Cre recombinase activity. Because transgenes are integrated randomly into the genome and usually in tandem repeats of variable copy numbers, it is difficult to assess the causes of the different expression levels in the testis observed in these two studies. One possible solution is to target a single copy of each of these transgenes to a specific locus, such as *Hprt* [32], for a more consistent genomic milieu for promoter analysis.

In summary, the present study provides an example of transcriptional regulation in germ cells. We have provided evidence to demonstrate that *cis*-acting sequences conferring oocyte-specific expression are present in the 3.3 kb of 5′-flanking sequences of m*Gdf9*. Our results also suggest that a

norvegicus; mouse, *Mus musculus*). **B**) Percentage identity plot (PIP) graph showing regional conservation between human and mouse *Gdf9*. Repetitive elements were masked during alignment. The genomic sequences were aligned using the DiAlign program (http://genomatix.de/), and the PIP graph was generated using the ECR Browser (http://ecrbrowser.dcode.org/).

testis-specific repressor element is located within a region 5.6 kb downstream of the *Gdf9* translation initiation site. In addition, we identified an evolutionarily conserved E-box at −182 to −177 bp upstream from the ATG site in mouse *Gdf9* that is essential for *Gdf9* expression in ovary but not in testis. Furthermore, we identified a promoter region of *Gdf9* directing high expression in testis. The strong promoter activity in the testis resulting from the 3.3 kb region is of great value for researchers interested in overexpressing genes in spermatocytes and spermatids and for targeting gene expression to the male germ cells at the particular stages. Germ cells contribute to the blueprint of the next generation. Appropriate regulation of genes in sex-differentiated germ cells is of critical importance for reproductive function in males and females.

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EXHIBIT G

"Matzuk, Martin Matthew" <mmatzuk@bcm.tmc.edu>

09/22/2008 03:16 PM

To "Hong-Van M Le" <hmle@jonesday.com>
cc
bcc
Subject RE: contact information for Changning Yan

The last I heard, she was still living and working in Houston. Is there anyone at Tanox that knows her cell phone number? Good luck, Marty

-----Original Message-----
From: Hong-Van M Le [mailto:hmle@jonesday.com]
Sent: Mon 9/22/2008 9:36 AM
To: Matzuk, Martin Matthew
Subject: contact information for Changning Yan

Dear Dr. Matzuk:

We are handling a patent application in which Dr. Changning Yan is identified as one of the inventors. We need to contact her regarding the patent application, but we have not had much success. We are contacting you, because you co-authored a paper (Yan et al., Biol Reprod. 2006 Jun;74(6):999-1006) with Dr. Yan. If you have any information regarding Dr. Yan's current contact information, we would appreciate it if you could provide us with that information.

The last address we have for Dr. Yan is a Houston address, and we have not been able to get a response writing to her yahoo email address (changnin_yan@yahoo.com).

Thank you for your time.

Best regards,

Van

Hong-Van M. Le, Ph.D.
Intellectual Property Legal Intern
Jones Day
222 East 41st Street
New York, NY 10017-6702
Tel: 1-212-326-3786
Fax: 1-212-755-7306

EXHIBIT H

"Huang, Dan" <dhuang@medarex.com>

09/23/2008 05:20 PM

To "Hong-Van M Le" <hmle@JonesDay.com>

cc

bcc

Subject RE: Dr. Changning Yan's contact information

Hi Van,

Thank you for confirming to receive my signature page. I don't have more updated information about Changning Yan because I left the company 4 years ago. Have you tried all the other people with their names in this patent? Hopefully someone will know her current address.

Dan

From: Hong-Van M Le [mailto:hmle@JonesDay.com]
Sent: Tuesday, September 23, 2008 2:40 PM
To: Huang, Dan
Subject: Dr. Changning Yan's contact information

Genentech Ref.: P4096R1-US
Jones Day Ref.: 12279-187-999
CAM: 403545-999187

U.S. Patent Application No. 10/583,927
National Stage of International Application No. PCT/US2004/43501
International Filing Date: December 23, 2004
Inventors: Fung et al.
Entitled: NOVEL ANTI-IL3 ANTIBODIES AND USES THEREOF

Dear Dr. Huang:

I confirm that I have received your executed declaration in connection with the above -identified application. Thank you very much!

I have been unsuccessful trying to contact Dr. Changning Yan, one of the identified co-inventors of the application. I have sent her several e-mails, but I have not received a response. The last address for her that I have in our files is a Houston address. I have also asked Dr. Michael Fung and Dr. Mason Lu, but they were only able to confirm that they have the same e-mail address that I have for her (changnin_yan@yahoo.com). If you have a cell number or any leads that may be helpful, please let me know. Thank you.

Best regards,

Van

Hong-Van M. Le, Ph.D.
Intellectual Property Legal Intern

Jones Day
222 East 41st Street
New York, NY 10017-6702
Tel: 1-212-326-3786
Fax: 1-212-755-7306

EXHIBIT I

JONES DAY

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Genentech Ref.: P4096R1-US
Jones Day Ref.: 12279-187-999
CAM: 403545-999187

September 23, 2008

Direct Number: (212) 326-3786
hmle@jonesday.com

Via UPS

Dr. Changning Yan
1800 El Paseo, #306
Houston, Texas 77054

Re: U.S. Patent Application No. 10/583,927
National Stage of International Application No. PCT/US2004/43501
International Filing Date: December 23, 2004
Inventors: Fung et al.
Entitled: NOVEL ANTI-IL3 ANTIBODIES AND USES THEREOF

Dear Dr. Yan:

Further to my e-mails of September 12, 2008, September 17, 2008 and September 21, 2008 regarding a Notice of Defective Response ("Notice") issued by the United States Patent and Trademark Office ("USPTO") on August 29, 2008 in connection with the above-identified application, in which you are identified as an inventor, we enclose herewith a Declaration of the inventors for the above-identified patent application for your signature.

The Notice requires that we submit a new Declaration by the **non-extendible** due date of **September 29, 2008**. The Declaration also has been sent to the other co-inventors for execution.

If the information for you in the enclosed Declaration is correct, then please ***sign*** and ***date*** the Declaration in box 204, and return a copy of the executed Declaration to my attention, preferably by e-mail or fax, before **September 26, 2008** so that we may file it in the USPTO by the September 29, 2008 deadline.

If the information for you in the enclosed Declaration is incorrect, then please contact me ***immediately*** and provide me with the correct information so that I can prepare a revised Declaration for your signature.

If you have any questions, please do not hesitate to contact me. Thank you for your assistance in this matter.

Sincerely,

Hong-Van M. Le, Ph.D.
IP Legal Intern

Enclosure

cc: Elinor Shin, Ph.D., Esq.
Jacqueline Benn, Ph.D., Esq.

HONG-VAN LE
2123263939
JONES DAY
222 EAST 41ST STREET
NEW YORK NY 10017

1 LBS 1 OF 1

SHIP TO:
DR. CHANGNING YAN
9999999999
1800 EL PASEO, #306
HOUSTON TX 77054

TX 775 9-03

UPS NEXT DAY AIR 1

TRACKING #: 1Z 104 45E 25 9572 3443

BILLING: P/P

Reference No.1: 403545-999187
Reference No.2: JP010202

XOL 02.11.14 NV45 82.0A 08/2008

TM

FOLD on this line and place in shipping pouch with bar code and delivery address visible

1. Fold the first printed page in half and use as the shipping label.
2. Place the label in a waybill pouch and affix it to your shipment so that the barcode portion of the label can be read and scanned.
3. Keep the second page as a receipt for your records. The receipt contains information useful for tracking your package.

EXHIBIT J

Close Window

Tracking Summary

Tracking Numbers

Tracking Number:	1Z 104 45E 25 9572 344 3
Type:	Package
Status:	**Delivered**
Delivered On:	09/24/2008 10:04 A.M.
Delivered To:	HOUSTON, TX, US
Signed By:	HERNANDEZ
Service:	NEXT DAY AIR

Tracking results provided by UPS: 09/26/2008 2:40 P.M. ET

NOTICE: UPS authorizes you to use UPS tracking systems solely to track shipments tendered by or for you to UPS for delivery and for no other purpose. Any other use of UPS tracking systems and information is strictly prohibited.

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EXHIBIT K

Matthew Moyle
<MMoyle@spaltudaq.com>

09/25/2008 04:30 PM

To Hong-Van M Le <hmle@JonesDay.com>
cc
bcc
Subject RE: U.S. Patent Application No. 10/583,927 - Dr. Changning Yan's contact information

History: This message has been replied to.

Dear van – Sorry, can't help with this one as I have been out of touch with her for >3 yrs. She lived south of Houston and may have been affected by the hurricane. Michael Fung would be most likely to know her most recent contact info.
Best,
Matthew

From: Hong-Van M Le [mailto:hmle@JonesDay.com]
Sent: Thursday, September 25, 2008 1:27 PM
To: Matthew Moyle
Subject: RE: U.S. Patent Application No. 10/583,927 - Dr. Changning Yan's contact information

Genentech Ref.: P4096R1-US
Jones Day Ref.: 12279-187-999
CAM: 403545-999187

U.S. Patent Application No. 10/583,927
National Stage of International Application No. PCT/US2004/43501
International Filing Date: December 23, 2004
Inventors: Fung et al.
Entitled: NOVEL ANTI-IL3 ANTIBODIES AND USES THEREOF

Dear Matthew:

In connection with the above-identified application, would you by any chance know Dr. Changning Yan's current contact information (e.g., address, phone number, e-mail, etc.)?

I have been unsuccessful trying to contact Dr. Changning Yan, one of the identified co-inventors of the application. I have sent her several e-mails, but I have not received a response. The last address for her that I have in our files is a Houston address (1800 El Paseo, #306). I have also asked Dr. Michael Fung, Dr. Mason Lu, and Dr. Dan Huang, but they were only able to confirm that they have the same e-mail address that I have for her (changnin_yan@yahoo.com). I also contacted Martin Matthew Matzuk, the last author in her latest scientific publication, but he didn't know her contact information. Any leads you can provide would be much appreciated. Thank you for all your help.

Best regards,

Van

Hong-Van M. Le, Ph.D.

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